THE NODULE ORGANISM OF THE LEGUMINOSÆ.

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(Plates li.-lii.)

It has been for a long time known to agriculturists that a leguminous crop enriches the soil to a considerable extent, and it is customary to sow a crop of beans, clover, or other leguminous plant as a preparation for wheat, which makes a great demand upon the soil nitrogen. It was the general feeling that the Leguminosæ could gain nitrogen from the air, but how this occurred was not understood.

In the middle of the century Boussingault, Villes, Lawes, Gilbert and Pugh studied the question, and although Villes certainly showed a gain of nitrogen in some of his plant experiments, yet the later investigations of the Rothamsted experimenters showed that neither the Leguminosæ nor any other plant could utilise the nitrogen from any source other than the soil. With the exception of Berthelot, who about 1876 doubted this conclusion, the matter lay practically dormant until Hellriegal and Wilfarth in 1886 published their classical researches upon the fixation of nitrogen. These authors showed that when crop plants were grown with a sufficiency of minerals the produce was proportional to the amount of nitrogenous manure in the soil. This law, however, did not hold for the Leguminosæ, which grew independently of nitrogenous manuring; indeed some of the largest crops of peas were obtained from soils which had received no nitrogen whatever. But they also showed that when the leguminous plant reached the "sick" period—that is, when the growing plant had exhausted all the cotyledonary nitrogen and appeared pale green in colour—it either took on a new lease of life or died, depending upon whether nodules appeared upon the roots. With the death of the plant there was no formation of nodules and no

gain of nitrogen, while with the survival over this sick period the nodules appeared, and there was a considerable gain of nitrogen. From this the inference was naturally drawn that leguminous plants could gain their nitrogenous food by absorbing the atmospheric nitrogen in some way, and that this action had an intimate relation with the nodules formed upon the roots. In other words, the nodules were capable of elaborating gaseous nitrogen into nitrogenous forms capable of being assimilated by the plant.

Hellriegal and Wilfarth in this way indirectly proved the fixation of nitrogen by showing that the mature plant contained more nitrogen than was originally in the soil. Schloesing and Laurent afterwards proved the fact directly by a loss of the atmospheric nitrogen in contact with the plant. Woronin, Marshall Ward and Frank had shown that the nodules did not form on the roots when the plants were grown in either sterilised soil or water, and it was only when the sterile soil was infected with ordinary soil, or when the plants in water culture had pieces of chopped nodules inserted between the root hairs, that nodules were produced. Woronin, as early as 1866, had suggested the presence of bacteria in the nodular tissue, and the earlier experiments bore out the idea.

Marshall Ward was the first to describe the entry of the organisms into the tissues of the plant through the root hairs. A bright spot was observed on the outer epidermal cell wall of the root hair; this fused with the cell wall, and emerging on the inner side, grew along the inside of the hair as a filament which reached the deeper layers of the cortex cells, and these by their proliferation ultimately formed the nodule. Since infection only occurs on the root hair the location of the nodule is accidental. The interior of the nodule is occupied by albuminoid cells, where the cellulose-dissolving infecting thread can be seen branching and passing like a mycelium from the protoplasm of one cell through the cell wall into the protoplasm of a neighbouring cell. The method of entry of the organism was confirmed by Prazmowski, who further saw a number of rods inside the simple filament of

Ward. Maria Dawson* showed that the filaments consisted of strands of straight rodlets imbedded in a matrix, the rodlets being heaped up at the places where the filaments are swollen. The rods appear to be liberated in the cell by the protecting mucilaginous or gelatinous membrane of the filament becoming dissolved, or by the bacteria budding off like a *Dematium*. Mazé agreed with the former alternative. When the bacteria become free they soon lose their original rod-like shape, becoming branched and stouter, and in this condition are known as bacteroids. The bacteroids may slowly fuse, one with the other, to form a spongy tissue, to which Beijerinck ascribed the fixation of nitrogen, likening it to the spongy tissue of the animal lung, where in one case there may be a fixation of nitrogen and in the other there is a fixation of oxygen. Beijerinck in 1888 announced that he had succeeded in isolating what he considered to be the infecting organism. His method of procedure was to sterilise the nodule by treatment with alcohol followed by ether, then to smash it up in a mortar with sterile water and to spread a few drops of the emulsion on plates, upon which a gelatine medium had been poured and allowed to set. The medium was made by adding 18 per cent. gelatine, $\frac{1}{2}$ per cent. peptone, $\frac{1}{4}$ per cent. asparagine, and 1 per cent. saccharose to an infusion of leguminous stalks and leaves. The solidified gelatine quickly absorbed the water, leaving the organisms upon the surface. After some days colonies were seen, consisting of short rods and motile swarmers, which might migrate from the parent colony to found a new colony at some distance. The organism, which he named *Bacillus radicicola*, appears to be pleomorphic, since it occurs not only as rods and minute swarmers but also develops branched forms, among which a simulation of the Greek letter γ is very common. A year later Prazmowski succeeded in infecting leguminous plants with pure cultures of the organism, the name of which he changed to *Bacterium radicicola*, since it did not appear to be capable of forming spores.

* Maria Dawson—Proc. Roy. Soc. lxiv., 167.

Beijerinck could not prove a fixation of nitrogen by pure cultures of the organisms when grown in artificial media, but he remarked that it could grow in the presence of a minimum quantity of nitrogenous material provided a sufficient amount of carbohydrate food was present. At a later date he advised the use of 8-9 per cent. washed gelatine, 2 per cent. sucrose with leguminous plant extract. With regard to carbohydrate food, it is to be noted that there is a considerable quantity of starch in the bacteroidal cells. *Bact. radicicola* in artificial culture is unable to fix nitrogen directly, but in the presence of carbohydrates it is able to seize the smallest trace of nitrate or ammonium salt and convert it into an albuminoid form. The bacteria separated from the nodules of the different genera of Leguminosæ differed in a slight degree, and although this difference prevented the bacteria from one genus producing nodules on the roots of other genera it was not sufficient to make one consider the bacteria as belonging to different families; they could only be considered as varieties of one species. Nobbe considers* that the organism is so influenced by the host plant that it becomes adaptable for existence only in that genus of plant.

As far as can be gathered,† the morphological and cultural characters of *Bacterium radicicola* as described by Beijerinck are as follows :—

Small motile swarmers $0{\cdot}18 : 0{\cdot}9\mu$, or non-motile rods $1 : 4\text{-}5\mu$; the rods show branching forms like the bacteroids of the nodules. No spore formation has been observed, and cultures are killed by exposure to 60°-70° C. The swarmers are strongly aerobic. Drying and freezing are without influence. Gelatine-, starch- and cellulose-dissolving or saccharose inverting enzymes are apparently not secreted. On gelatine the colonies grow slowly, are hemispherical, whitish, clear or somewhat turbid; the smaller colonies

* Stutzer, in Centralblatt für Bakteriologie, 2 Abt. i. 68.

† Lafar, Technische Mykologie.
Kruse, in Flugge's Die Mikroorganismen.
Beijerinck, Centralblatt für Bakt. 1 Abt. v. 804

are firm and adhesive; the larger are watery. The gelatine is not liquefied.

Frank,* under the name *Rhizobium leguminosarum*, describes an organism which seems to be Beijerinck's bacterium. The rhizobia are actively motile, rounded to long in shape, and 0·9 to 1·3 μ in length. There are also non-motile forms; flagella, however, could not be found. Curved forms, more or less constricted in the middle, apparently a division stage, were frequently observed. Zoogloea forms also were seen, and these often contained in the gelatinous matrix very short coccus-like bodies, the size of which was estimated at 0·2 μ. Spores were not observed. On gelatine the colonies grow slowly, reaching a diameter of 1 mm. in about a week. They are small, rounded to elliptical, raised, of a pale yellow colour and mucilaginous. The gelatine is sometimes liquefied.

Kirchner† claims the organism of the Soja Bean as a variety of *Rhizobium*. The rods are generally somewhat bent, and measure 0·8 : 3·2-3·6 μ. They show a granular content when stained, and are non-motile. On gelatine the colonies grow slowly, forming raised, rounded, transparent, white paraffin-like drops which do not liquefy the medium. Laurent,‡ in discussing the organism of the nodule, prefers the designation *Rhizobium leguminosarum*, but he differs from Frank in respect to its morphological characters. The colonies on gelatine are whitish, and have a glistening surface. The strongly developed colonies are slimy, the slime staining well with dahlia violet, yellow with iodine, and shows no cellulose reaction. It thrives well in media destitute of nitrogen. Sugar, especially saccharose, is favourable. When the medium is 5 mm. deep, a slimy precipitate is formed; when 1 cm. deep floccules are obtained, and with deeper layers there is only a turbidity. The medium should be neutral or slightly alkaline.

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* Frank, Centralblatt fur Bakt. 1 Abt. IX. 629.

† Kirchner, *ibid.*, 2 Abt. II. 96.

‡ Laurent, *ibid.*, 1 Abt. IX. 703.

Temperatures from 22°-26° are most favourable; the growth ceases at 30°. In bouillon, a slimy precipitate is formed, which consists of rods and branched forms. Motility could not be observed even in the smallest forms.

Beijerinck,* from the nodules of *Vicia lathyroides*, obtained a species of *Bact. radicicola*, which in artificial media had a pronounced capsule, forming threads and balls similar to the appearances seen in nodule sections of some genera of *Leguminosæ*. In the capsule the rods do not assume the bacteroidal form.

Gonnermann† considered that the bacteriological research of the nodule had been kept in the background; those who had investigated the nodule question had done so from a botanical and an agricultural-chemical point of view. Beijerinck had not described his bacterium at all fully; indeed, he mentioned the organism as being ciliated, although he had not observed the flagella. As a result of his own researches, Gonnermann did not consider the nodule to be produced by the stimulus of one organism alone, but to result from the action of several. Out of nine bacteria which he separated from sterile soil, in which nodules had been produced on plants by infection with cut nodules,‡ he found two cocci which by themselves were capable of producing nodules on leguminous roots. This is the first intimation that cocci may produce the nodules, although Frank spoke of cocci which became bacteria in the tissues, and Beijerinck claimed that *Bact. radicicola* may assume the coccus, bacterium or spirillum form.

Klein§ claims to have proved nodule-formation on the lupin by two bacteria, one allied to *Bacterium fluorescens liquefaciens*, and the other a short, oval, non-motile bacterium, which stains deeply at the ends and produces small colonies that slowly liquefy the gelatine.

It is evident that the bacterial flora of the leguminous nodule may be very varied—a circumstance which is to be expected by

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* Beijerinck, *Ibid.*, xv. 728.

† Gonnermann, Centralb. für Bakt. 2 Abt. i. 200.

‡ Kruse—Flugge, Die Microorganismen.

§ Klein, Centralb. für Bakt. 1 Abt. xvi. 840.

all who have done bacteriological work with plant tissues, for it is a matter of general experience that many bacteria which live in the soil obtain access to the plant. Galippe* found that garlic was the only plant the tissues of which were free from soil bacteria. There should not, however, be so much doubt with regard to the bacteria which cause the formation of the nodule; neither should the morphological and cultural characters of the organism be so indefinite. Of the bacteria for which nodule-forming power is claimed, there is distinct evidence in favour of two, viz., *Bacterium radicicola*, Beijerinck, and *Rhizobium leguminosarum*, Frank. The differences between these two organisms are not very great, and it is probable that were the two examined by one bacteriologist they would be found to be identical. The differences certainly do not justify a difference in name, especially with a microbe which is admitted to be on the borderland between the bacteria, the saccharomycetes and the hyphomycetes. Each investigator considers it to be allied to a different family, and an organism, the characteristics of which are so different from the bacterial type, should have a specific name. The appellation, therefore, given by Frank is to be welcomed, especially as it is becoming more evident that the name bacterium or bacillus must be retained for those organisms that are of a fixed type. Those that grow like the hyphomycetes in some of their stages are now being called by names which indicate a variance from the true type of the fission fungi.

The circumstance that gives the nodule bacterium its interest is undoubtedly the fact that it either fixes atmospheric nitrogen itself or stimulates the plant to do so. Both Beijerinck and Frank state that pure cultures of their organisms do not assimilate free nitrogen. Heindrich also showed that the organism grew well on sterile potato, but did not fix nitrogen. On the contrary, Mazé† obtained a decided gain of nitrogen in bean sucrose media, containing 1 part of nitrogen and from 100 to 200 parts of sucrose.

* Galippe, Centralb. fur Bakt. I Abt. III. 108.

† Mazé, Annales de l' Institut Pasteur XII.

Other investigators claim, as the results of experiments with growing plants, that fixation only begins when the bacteria have become degenerated in the nodular tissue into bacteroids. As long as they exist in the rod-form there is no fixation. While this seems true for the plant and the bacteria, Liebscher and Prazmowski think that *Bact. radicicola* can fix nitrogen in the soil, and Stutzer suggests that other bacteria may assist. This is quite possible, for such a fixation has been shown with other bacteria and minute plants. Schloesing and Laurent* obtained nitrogen assimilation with certain algæ and mosses growing upon the surface of soil. Winogradsky† separated from soil a bacterium which, together with two other species, gained a notable quantity of nitrogen when cultivated in a nitrogen-free glucose medium. This is an interesting case of company-working among bacteria.

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In order to give the organism the food constituents which are presumably required for its growth, an extract of some leguminous plant is made, and this is used as a basis, in the same way that meat extract forms the basis of media for the growth of bacteria parasitic in animals. In this investigation the lupin was first examined, and consequently this plant was employed. A kilogram of chopped stems and leaves was boiled with a litre of Sydney town water for several hours, and then pressed through a meat press. The resulting extract was evaporated to less than a litre, filtered and made up to the volume. In the beginning of the experiments a simple agar medium was prepared by adding 2 per cent. agar to the infusion, and after the usual methods of procedure, 10 cubic centimetres were caused to set in Petri dishes. Several young lupin plants were dug up, the nodules washed, cut off, and the outside sterilised by steeping for 15 minutes in mercuric chloride (1-1000), then for a minute in strong spirit, followed by

* Schloesing and Laurent, Journal of the Chemical Society, lxii. Abs. II. 1021, and lxiv. Abs. II. 138, 336.

† Winogradsky, Centralb. für Bakt. I Abt. XVI. 129.

half a minute in ether. The nodules were picked out of the ether, held with the forceps till the ether had evaporated, and cut open with a sterile knife. The cut surface was rubbed over the solidified agar in the Petri dish. After several days' incubation at 22° C. many growths appeared on the plates, but in none of them could the typical organisms be observed. This is not extraordinary, for Marshall Ward complained that it was not so easy to obtain a culture from the nodules as the description of Beijerinck would lead one to believe.

There is a considerable difference of opinion with regard to the medium best suited to the organism. Beijerinck in his later papers recommended a very poor medium, and ascribed the want of success that experimenters had experienced in their endeavours to obtain the organism, to the employment of media rich in albuminoids. Atkinson found that it grew well in ordinary meat agar. Gonnermann used a plant infusion with 3 per cent. peptone. Mazé recommends a plant extract with 3 per cent. saccharose. Beijerinck did not neutralise the natural acidity of the extract, while Laurent and also Mazé advised a neutral or slightly alkaline medium.

In the plates containing the simple unneutralised medium, no colonies of the organism could be obtained, but after about a week a dark coloured smudge was noticed on one of the plates. An examination of this slight stain showed a few irregular forms of the organism, and several tubes of different media were inoculated. The only medium in which growth took place was one recommended by Hansen for cultivating yeast. As advised by him, however, it is too acid, and consequently it was neutralised.* The culture in the faintly acid medium was purified by inoculating a series of three liquefied ordinary nutrient gelatine tubes, and

* The peptone-glucose medium eventually used contained :—Peptone, 10 grams ; glucose, 50 grams ; calcium chloride (cryst.), 5 grams ; monopotassium phosphate, 2·5 grams ; tap water, 1000 c.c. Neutralise with caustic potash until 10 c.c. contain an acidity equal to 0·7 c.c. tenth normal acid. Boil, filter and sterilise.

pouring these into Petri dishes. In about 10 days colonies grew on one of the plates to a millimetre in diameter. Different media inoculated from one of the colonies showed the following characteristics when grown at 22° C. :—

Meat-gelatine plate.—The surface colonies appear as raised hemispheres with a white, glistening, paraffin-like appearance; glutinous when touched with the needle. With 60-fold magnification they are circular and opaque except at the margins where a little light passes through showing a granular structure. The deep colonies are oval or round, brownish and coarsely granular.

Stab cultures in various gelatine media.—White uncharacteristic growth along the needle track; slight surface growth.

Lupin-agar with 1% potassium chloride.—Luxuriant, stearine-like growth which has extracted some of the colour of the medium.

Meat-agar stroke.—The inoculating loop has produced a thin, rough, glistening, whitish ribbon with rough margins; the culture gravitates slightly to lower portions; growth never luxuriant.

Glycerine-meat-agar stroke.—At first the growth is like that on meat-agar, later it becomes more luxurious. In three weeks there is an exceedingly voluminous raised, spreading, white glistening culture.

Inorganic fluid media.—Scanty growth.

Peptone-glucose fluid media.—Turbid with slight film and flocculent precipitate.

Peptone-sucrose fluid media.—Clear with film and precipitate chiefly of old films.

Potato, ordinary acid.—A yellowish-white, spreading, glistening layer.

Lupin-extract, etc., gelatine plate —Translucent, white, raised, non-spreading colonies. With 60-fold magnification, circular granular colonies with sharp margin; the deep colonies are like the surface ones, but are more opaque, and consequently appear more granular.

The earlier cultures in agar media made from the unneutralised infusion were not at all successful, a circumstance due partly to the acidity and partly to the agar surface which was very soft owing to the action of the acid which, as sterilisation proceeded, made the medium less and less gelatinous. This was obviated by neutralising the medium immediately after the agar or gelatine was dissolved. Potassium hydrate suggests itself as the best alkali to use in neutralising a plant extract, especially when one remembers how much the Leguminosæ are benefited by potash salts. In some of the cultures, as for example lupin-agar, with 1 per cent. potassium chloride, it seemed as if the salt had stimulated the growth of the organism. According to Mazé, sodium chloride acts as a poison towards the nodule bacterium paralysing its development. A plate seeded with the organism and dotted with solutions of various salts showed the greatest amount of growth between a potassium phosphate and a calcium chloride manuring. This suggested a means of clarifying the various plant-extract media which are always more or less turbid from the gradual precipitation of organic matter. When the agar or gelatine is dissolved in the plant extract 5 c.c. each of a 10 per cent. solution of monopotassium phosphate and of a 20 per cent. solution of crystallised calcium chloride are added to every 100 c.c. of the hot gelatine or agar medium, which is then neutralised with 10 per cent. potassium hydrate to faint acidity. Ten c.c. of the solution are pipetted out and neutralised with tenth normal potash, using phenolphthalein as an indicator, and normal potash is added to the bulk of the medium in proportion to make every 100 c.c. possess an acidity equal to 0·7 c.c. of normal acid. This acidity is equal to 0·05 per cent. tartaric acid.

The organism is a strong aerobe, and grows most freely when started upon the surface of a medium. It does not grow under anaerobic conditions in peptone-glucose fluid, a medium which seems best suited to its needs. Laurent maintained that it could grow anaerobically, while Mazé, denying this, assumed that oxygen had not been thoroughly eliminated from Laurent's culture media.

Ordinary acid potato forms an excellent medium for its growth, and yet it refuses to grow upon a medium prepared by adding 2 per cent. starch and 2 per cent. agar to acid potato extract. The failure of the organism to grow upon this medium cannot be due to the acidity, for the steamed potato and the potato-agar had about the same acidity. It is more probable that starch is not the carbohydrate in the potato that is utilised, and that in the nodule the organism does not utilise the starch as such. Steaming possibly alters some of the relatively great amount of starch in the acid potato into a derivative, which can supply the organism with carbohydrate food. This derivative cannot be dextrin, for experiment showed that when dextrin is added to ordinary meat-agar to the extent of 5 per cent. it retards the growth. Extract of lupins or of other leguminous plants does not seem a necessity for the culture media. Grass will do quite as well, and for that matter the plant extract might be left out entirely. Fairly luxuriant cultures were obtained upon a medium made with 10 per cent. washed gelatine, 3 per cent. glucose, and the customary calcium chloride and potassium phosphate. The most luxurious growth was obtained with meat-agar containing 6 per cent. glycerine. More than this percentage of glycerine, *e.g.*, 10 per cent. or 20 per cent., prevented growth.

With regard to temperature, the organism grows very well at 22° C., and this is very fortunate since it enables gelatine media to be employed. At 30° C. growth is slow, but it is by no means checked. Mazé was able to accustom the organism to grow at 35° C.

The media ultimately adopted were peptone-glucose as a fluid (see footnote, p. 661), and glucose-glycerine agar or gelatine as a solid.*

* Washed gelatine, 20 grams, or washed agar, 2 grams; lupin extract, 100 c.c.; glucose, 4 grams; glycerine, 2 grams. Heat until the gelatine or agar is dissolved, add 10 c.c. each of 10 per cent. monopotassium phosphate and 20 per cent. calcium chloride, make the volume up to 200 c.c. and neutralise until there is an acidity equal to 0·05 per cent. tartaric acid (*i.e.*, until 10 c.c possess an acidity equal to 0·7 c.c. tenth normal acid). Heat, filter, sterilise.

Although a culture of the nodule organism was obtained from the lupin nodules by smearing the surface of set agar, the method did not recommend itself as one at all well adapted for easily getting the organism.

Beijerinck's method of sowing drops of nodule emulsion was just as useless, because the places were in a few days swarming with other bacteria.

Better results were obtained by washing the nodules and passing them successively through mercuric chloride, alcohol and ether, holding them with sterile forceps until the ether evaporated and placing each into a Freudenreich flask containing 10 c.c. sterile, 0·6 per cent. potassic chloride. In the flasks the nodules were crushed with stout sterile glass rods. The emulsion thus obtained was blown by means of a sterile glass spray upon the surface of set gelatine medium in a Petri dish. From six to twelve plates should be prepared from the same number of nodules, as some of the nodules may contain foreign organisms which grow quickly and generally liquefy the gelatine. One objection to spraying the plates is that the air is washed at the same time, and moulds and aerial bacteria carried to the gelatine surface. The usual method of obtaining pure cultures by inoculating the gelatine, previous to pouring into plates, is not to be recommended, as the nodule-formers are then chiefly in the body of the gelatine film, and grow very slowly indeed, especially when taken directly from the nodule where they are presumably in a somewhat enfeebled condition. The passage through the potassium chloride seems to act as a stimulant, for the colonies grow faster than when distilled water is employed.

A better method than spraying consists in sterilising a small camel's-hair brush or pencil by passing it successively through mercuric chloride, alcohol and ether, allowing the ether to evaporate and washing in sterile potassium chloride. The moist sterile brush is then pushed about in the nodule emulsion and painted over the set gelatine surface. Confluent or isolated colonies appear in from six to ten days, and from these a pure culture

may be obtained in the usual way by inoculating a series of tubes and pouring into Petri dishes.

The colonies are circular, well raised from the surface and white. The white colour may give place to a yellowish from absorption of the colouring matter of the medium. In a pale coloured medium the colonies are like drops of paraffin or skimmed milk; on the same plate both yellowish and white colonies have been observed near one another. The yellowish was the older colony, and apparently had absorbed all the free colouring matter before the younger had made much progress. Although the colonies do not liquefy the gelatine, yet in some cultures a slight liquefaction has been seen. This was obtained with a vigorous culture growing upon a medium containing 6 per cent. gelatine which, through prolonged heating during filtration, had lost some of its gelatinising power. On the plates the colonies may consist of many forms of the organism. Some colonies may consist entirely of short bipolar staining rods in the interior as well as on the surface of the growth. Others again, even on the same plate, may consist of these together with rods swollen at the ends and exhibiting irregular staining, or with Y, saturn-like, or branching forms.

The organism, generally speaking, is a capsulated bacterium, with rounded ends and stains irregularly. The strong stains such as fuchsin, unless the excess of colour is removed by alcohol, show an irregular rod that may be more or less branched, while the weaker stains as the blues show the protoplasm contracted in places. The shorter bacterial forms are straight and stain at the poles; the longer forms may be more or less bent, and show three, four, five or more stained portions. The general shape varies somewhat in the different media. In peptone-glucose fluid the short bipolar staining rods predominate, while the substitution of sucrose for glucose causes the irregular and branching forms to preponderate. On ordinary meat-agar media the broken rods appear to be thin in the middle; the addition of glycerine to the meat agar causes some of the organisms to assume the long form, the segregated protoplasm of which gives the rod a chlamydospore-

like appearance. The broken appearance of the dried and stained rod is very characteristic.

A few of the films that had been made from peptone-glucose fluid cultures showed small terminal prominences that suggested buds, and in order to observe them better, the films, instead of being fixed by heat, as is customary in preparing bacterial films, were fixed by means of formalin, the employment of heat being avoided throughout the process. The method consisted in spreading a loop of a 36 to 48 hours culture upon a clean cover glass and allowing the film to dry in the air. It was then floated on a 5-10 per cent. aqueous solution of formalin for five minutes, rinsed in distilled water, floated on the stain, again washed in tap followed by distilled water, allowed to dry in the air and finally mounted in balsam. Of the various stains, gentian-violet used as Fränkel's carbol-violet gave the best result The blues were rather weak, and carbol-fuchsin stained the whole organism, although when diluted it did fairly well.

The organisms prepared in this way appeared as more or less oval vacuolated yeasts, and a few of the cells showed a pronounced terminal bud. The yeasts are undoubtedly best seen in the fresh condition, but the nodule organisms are much too small for observation in this way, and consequently the use of a differential stain is necessary. When prepared in this way the single cells vary in length and breadth, but generally are about 0·5 μ broad and from 1·2 to 2·0 μ long. The longer forms consist of several cells contained in a delicate tubular capsule. We can now explain the broken appearances of the organisms when prepared by the methods usually adopted for bacteria. The heat used to fix the organisms causes the protoplasm of the cell to contract, and a break occurs across the vacuole. The single organism thus exhibits polar staining. The organisms may have produced a bud more or less mature that separates from the parent cell, but is still retained within the capsule. The stained organism and bud will now appear as a rod, staining centrally and at the poles. The bud may mature and form its vacuole, in which case two organisms will be contained in one capsule. This double organism will stain

as a straight or bent rod, the protoplasm of which has collected in four places.

A hanging-drop preparation of a two days old culture in peptone-glucose fluid at 22° C. shows the young cell as actively motile, darting about over the field of the microscope. At a later stage it has a forward waltzing motion, and ultimately the motion ceases when the cell presumably begins to bud. When the bud has separated from the parent protoplasm it pulls and tugs in its endeavour to free itself from the capsule membrane containing the motionless mother cell, and we have an appearance exactly like that of an ant attempting to drag along a twig which proves too heavy for its powers.

The capsule is frequently too strong, and the bud grows to maturity still enclosed in the parent membrane. In young cultures budding is very vigorous, and a second bud may appear pushing the first to one side. Thus there is produced the Y form. Another bud may form an X.

In peptone sucrose media the irregular forms are very common; indeed with a two days' culture there are very few individual cells. These combinations clearly result from the inability of the daughter cells to escape from the parent membrane, which is apparently much more tough than when glucose is used as a nutrient. When grown upon solid media, the cells are generally in the rod form, but this does not justify their being placed among the bacteria. Indeed, since they are budding fungi, the name applied to them by Beijerinck is a misnomer.

A year ago Maria Dawson, by constant observation under high magnification, found that the organisms divided into equal or slightly unequal halves, but since they divided, this investigator considered that they were true bacteria. As before mentioned, the organisms are too small to be seen clearly in the unstained condition, and the observation of even the more mature buds is a matter of some difficulty. The younger buds enclosed in the refractile membrane are probably impossible to be seen until they have attained a more mature form, when they appear as if division had occurred.

The nodule yeasts have always a tendency to form a more or less gelatinous capsule. In peptone-glucose fluid this is very thin, while in solid media it is more or less bulky. Under some conditions, and notably in sucrose fluids, the cells are collected in zoogloea films, tufts and filaments. They are very prone to collect round foreign solid particles, such as fragments of cotton wool, and when this occurs there is presented the appearance of a microscopically wide tube containing the organisms. The capsule, when swollen and mucilaginous, gathers more or less towards the middle of the simple cell, or of the elongated or branching compound cells, and by staining equally with the cell produces many odd forms. Among these odd forms there is a lenticular shape, and a sphere with two or three projecting points: the two projecting points cause the organism to appear like the planet Saturn. The other varieties of form may be called hat-shapes. These irregular appearances are only observed when stains are used that colour the capsule as deeply as the cell. The relation between the capsule and the organism may be demonstrated by staining with carbol-fuchsin, and washing most of the stain out of the capsule with dilute alcohol. The cell then appears of a deep red colour, and the capsule pink.

In my endeavour to obtain a preparation showing the flagellum by means of which the cell presumably is enabled to move about, many cultures of the organism were tried in various ways. As a result of these trials it became evident that the suspension of an agar culture in water or normal saline was not suitable. Ultimately peptone-glucose fluid cultures were used in the undiluted condition, spread on clean cover-glasses, air-dried and fixed in 5-10 per cent. formalin solution. The formalin solution, while fixing the organisms, probably also extracts some of the soluble constituents of the film which might take up the mordant and become stained. The formalin was washed off with distilled water, and the cover-glass immersed in Coerner-Fischer mordant that had been warmed and filtered. The watch-glass containing film and mordant was kept warm by placing it over the very small flame of a microchemical burner.

After from 1 to 2 minutes, the cover-glass was taken out of the solution, rinsed thoroughly in tap water and then in distilled water. Staining was effected by immersing the cover-glass, film-side downwards, for 5 minutes in carbol-fuchsin, which had been filtered cold and then warmed. The stained film was washed, air-dried and mounted in balsam. When successfully stained by this method the appendages of the cell are revealed. An empty tubular capsule can sometimes be seen attached to the organism; the width of the tube, as well as the frayed end, show clearly what it is. The cell has sometimes a relatively wide diffuse terminal thread, which is in all probability a mucilage thread and accidental, since it is too wide and transparent either for a flagellum or for the capsular tube. A few cells have stronger threads varying up to twice the length of the organism. These are exceedingly like the flagella of the bacteria. They may be flagella—it is more probable that they are not, since they are but seldom found. For example, in a 40 hours' culture at 18° C., most of the organisms were actively motile, and a film of this culture showed when mordanted and stained only two cells with these pronounced terminal threads. Had they been flagella there would have been in the same film many more cells endowed with these appendages. The culture, however, showed that practically every cell bore an exceedingly thin terminal thread varying up to 2 μ in length, and bearing upon the distal end a tuft like the tuft upon a lion's tail or the lash upon a whip. This is undoubtedly the flagellum by means of which the cell moves. The thread is so thin that even when mordanted and stained it is seen with difficulty. The terminal tuft, however, is easily made out, and assists in the discernment of the thread. The tufted flagella appear singly and at one end of the simple organisms.

While the coccus form of other investigators is undoubtedly the bud, the spirillum and slightly bent forms are caused by the bending of two or more cells while still enclosed in the parent membrane, and the collection of individual organisms appearing or staining as one bacterium produces the curvature of the supposed simple rod. It must not be forgotten, however, that in

common with all yeasts the rhizobia under certain unfavourable conditions, and notably within the nodule, may grow to long and irregular forms, just as some of the most pronounced saccharomycetes grow as sausage-shaped and lengthened forms. With the latter this frequently occurs when they are grown on solid media, and also when cultivated for a long time on the surface of liquid media.

When young cultures of *Rhizobium* are placed upon the gypsum block, as is customary in determining ascospore formation with the yeasts, and maintained for a few days at 22° C., the protoplasm of the cell is seen to aggregate into points and finally disappear, the cell meanwhile swelling and losing its staining power. Among the cells occur a number of coccus forms, but since they occur free, and have not with certainty been seen inside the cells, they are probably buds and not ascospores. The older cultures on gypsum show only a collection of non-staining forms.

Experiments were made with pure cultures of the organism, using glucose and sucrose in conjunction with plant extract, but neither with *Rhizobia* obtained from the lupin nor the pea could any fixation of free nitrogen be found either in faintly acid, neutral or faintly alkaline media; the cultures finally contained the same amount of nitrogen as they had at the beginning of the experiment.

With regard to the other organisms of the nodule, examination of the crushed nodule suspension shows what is virtually a pure culture of *Rhizobium*. Other organisms are so few in number that they are overwhelmed by the nodule formers. So numerous are they that any doubt as to whether other organisms may cause the formation of the nodule is at once dispelled, and *Rhizobium* undoubtedly plays the chief if not the only rôle. Other organisms do occur, but most of them may be looked upon as accidental, since they are not universally found in all nodules. There is one organism, however, which has been found very frequently in the nodules of peas, lupins and vetches. It grew so freely upon

carbonaceous media poor in nitrogen, and was of so large a size that experiments were made in order to ascertain if this could fix free atmospheric nitrogen. The experiments were negative; the blanks showed the same amount of nitrogen as the cultures. This organism appeared sometimes as a streptococcus, and sometimes as a chain of fat bacteria, the individual cells measuring about 3 μ long and about 2 μ broad. A culture in lupin extract that had stood for two months showed a collection of spores. On solid media these developed into smaller compact rods with rounded ends, and this appearance, together with the culture characteristics obtained from the original organism, identified the bacillus as *Bac. megatherium*. The recognition of this organism, which, if not identical with, is very closely allied to, the alinit bacillus, *Bac. Ellenbachii a*, which is claimed to assist the cereals in collecting nitrogen from the air, induced the trial of a mixed culture of this bacillus with *Rhizobium* in order to see if these organisms growing together could fix atmospheric nitrogen in artificial culture. The mixed culture grew most luxuriantly to form a syrupy fluid, which was in great contrast to the thinner cultures of the separate organisms. There was no gain of nitrogen, however, by the cultures. A second set received an additional quantity of glucose after reaching the syrupy stage, but still there was no gain. Cover-glass preparations of the eleven days' syrupy culture showed the rhizobia staining strongly as if in extremely vigorous condition. A number of short empty capsule tubes were dimly visible. The growth of megatherium was restricted; spores occurred here and there, and there were a few short chains of coccus forms. The small coccus-like buds, as well as the mature forms of *Rhizobium*, were frequently seen adhering to these chains. There were a few large oval cells which contained one or two rhizobia; the cells apparently consisted of a stain-absorbing plasma, and probably were huge capsules. Yellow masses of bye-product also occurred; these recalled the masses after seen in the nodule cells. Bearing in mind that the nodules are rich in starch, it seems possible that *Bac. megatherium* may functionate as a starch dissolver, and in this way assist the nutrition of *Rhizobium*.

Of the other bacteria and moulds of the nodule there are none that call for any special attention. When taken from the nodule they are chiefly capsulated gelatine-liquefying bacteria. *Bact. fluorescens liquefaciens* was obtained from the nodules of one pea plant in goodly amount; but since it was not found in any other, its presence was purely accidental. Stutzer's *Hyphomicrobium* occurs very frequently as an impurity in the partially pure colonies of the nodule former.

The following are the points which this investigation has decided :—

1. The nodule organism is a yeast and possesses a vacuole.
2. Frank's designation *Rhizobium leguminosarum* is better than Beijerinck's *Bacterium radicicola*.
3. The organism multiplies by budding, which, together with the presence of a more of less persistent mucilaginous capsule, causes the single or compound organism to assume a variety of shapes.
4. The vigorous forms are motile, the motility being due to a single, terminal, tufted flagellum.
5. A faintly acid glucose medium is best adapted to its growth.
6. The organism does not fix nitrogen in artificial media.
7. *Bac. megatherium* usually accompanies *Rhizobium* in the nodules.
8. Other bacteria found in the nodules are probably accidental.

EXPLANATION OF PLATES.

Magnification 1500. Culture medium, peptone-glucose fluid. Numbers 3-7 stained with Coerner-Fischer mordant.

Fig. 1.—Double cell and pronounced vacuole.

Fig. 2.—Groups of budding and vacuolated cells.

Fig. 3.—Budding cells.

Figs. 4-7.—Cells with flagellum appendages.

Fig. 8.- Cells in *Megatherium-Rhizobium* culture showing *Rhizobium* cells in large capsule and also in thin branching capsule.